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Transmitted herewith for filing is the patent application of

Inventor(s): **Judith W. Zyskind**

For: **CHITOBIASE AS A REPORTER ENZYME**

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For	:	CHITOBIASE AS A REPORTER ENZYME
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CHITOBIASE AS A REPORTER ENZYME

GOVERNMENT INTEREST IN THE INVENTION

Work performed in developing the present invention was supported, in part, by National Science Foundation grant MCB-9507209, NIH MBRS grant GM45765, and NIH NIGMS MARC F31 GM14967-0451. Accordingly, the United States Government may have certain rights in the invention.

Related Applications

This application claims priority to U.S. Provisional Patent Application Serial Number 60/159,221, filed October 13, 1999, the disclosure of which is incorporated herein by reference in its entirety.

Background of the Invention

Field of the Invention

Reporter enzymes are enzymes whose activities are easily assayed when present inside cells. In order to study the regulation of a gene whose expression is regulated by various environmental and/or cellular factors or influences, a gene encoding a reporter enzyme may be fused to the coding region or to the regulatory region of the regulated gene.

One of the most popular cytoplasmic reporter enzymes for use in bacteria is ß-galactosidase. It is widely used in the art; however, because bacteria such as *Escherichia coli* contain an endogenous ß-galactosidase encoding gene and ß-galactosidase may be present in the cytoplasm of such bacteria, deletions of the LacZ gene, the source of the enzyme, must be introduced into the host cell line prior to its use. One goal of the present invention is to provide an alternative intracellular enzyme for use as a reporter.

This invention relates to genetic constructs and methods of using a cytoplasmic form of the chitobiase enzyme as a reporter. The invention also comprises expression vectors which express the cytoplasmic form of chitobiase. As used herein, all instances of the terminology "chitobiase" refer to a form of chitobiase which is present in the cytoplasm of the cell. Cytoplasmic forms of chitobiase may be generated via genetic engineering or microbial selection techniques.

Summary of the Invention

One embodiment of the present invention is a method for characterizing a promoter comprising providing a construct comprising the promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and identifying sequences in the promoter which regulate transcription levels. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein which comprises a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In still another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In a further aspect of this embodiment, the method of identifying sequences which are involved in directing transcription comprises mutagenizing the promoter. In another aspect of this embodiment, the method of identifying sequences which are involved in transcription comprises constructing deletions in the promoter.

Another embodiment of the present invention is a method for identifying a regulatory element capable of directing or regulating transcription within a test nucleic acid sequence comprising providing a construct comprising the test nucleic acid sequence operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and determining the level of chitobiase activity. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, the fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In still another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a

cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In one aspect of this embodiment, the reporter gene construct is introduced transiently. In another aspect of this embodiment, the reporter gene construct is introduced stably. The host cells may be selected from the group consisting of prokaryotic cells and eukaryotic cells. In another aspect of this embodiment, the method further comprises permeabilizing or lysing the host cells. The permeabilizing or lysing step may comprise treating the host cells with toluene. The step of determining the level of chitobiase activity may be selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate. In another embodiment, the step of determining the level of chitiobiase activity may comprise determining the level of *p*-nitrophenol released from a substrate. In another aspect of this embodiment, the test nucleic acid sequence comprises a portion of genomic DNA. In a further aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing the host cells to a desired set of environmental conditions. In still another aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting the host cells with a compound to be tested for its influence on the level of transription from siad regulartory element.

Another embodiment of the present invention is a method of detecting successful transformation, comprising the steps of introducing a nucleic acid encoding a cytoplasmic form of chitobiase into host cells, and detecting chitobiase expression in the host cells.

Another embodiment of the present invnetion is a fusion protein-reporter gene construct comprising a promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase fused in frame with a nucleic acid encoding a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof, and wherein the heterologous polypeptide does not contain a signal peptide. In one aspect of this embodiment, the nucleic acid encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In another aspect of this embodiment, the nucleic acid further comprises a λ site-specific recombination sequence.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pJMF3.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pJMF4.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pDYK9.

Another embodiment of the present invention is a reporter gene construct comprising plasmid pDYK11.

Another embodiment of the present invention is a host cell comprising a fusion protein-reporter gene construct comprising a promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase fused in frame with a nucleic acid encoding a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof, and wherein the heterologous polypeptide does not contain a signal peptide. In one aspect of this embodiment, the nucleic acid is integrated into a chromosome of the cell. In another aspect of this embodiment, the nucleic acid is transiently expressed in the host cell.

Another embodiment of the present invention is a nucleic acid encoding a cytoplasmic form of chitobiase in which the signal sequence of native chitobiase has been inactivated or deleted. In one aspect of this embodiment, the signal sequence has been mutated to inactivate it.

An isolated or purified polypeptide comprising a cytoplasmic form of chitobiase fused in frame with a heterologous polypeptide, wherein the heterologous polypeptide is not β-galactosidase or a portion thereof and wherein the heterologous polypeptide does not contain a signal peptide.

Another embodiment of the present invention is an isolated or purified polypeptide comprising a cytoplasmic form of chitobiase in which the signal peptide of native chitobiase has been inactivated or deleted. In one aspect of this embodiment, the signal sequence has been mutated to inactivate it.

Another embodiment of the present invention is a method for monitoring the activity of a promoter comprising providing a construct comprising the promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase, introducing the construct into host cells, and determining the level of chitobiase activity. In one aspect of this embodiment, the cytoplasmic form of chitobiase lacks a signal sequence. In another aspect of this embodiment, the nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, the fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide. In one aspect of this embodiment, the nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*. In another aspect of this embodiment, the reporter gene construct is introduced transiently. In still another aspect of this embodiment, the reporter gene construct is introduced stably. In a further aspect of this embodiment, the host cells are selected from the group consisting

of prokaryotic cells and eukaryotic cells. In yet another aspect of this embodiment, the method further comprises permeabilizing or lysing the host cells. For example, the permeabilizing or lysing step may comprise treating the host cells with toluene. In yet another aspect of this embodiment, the step of determining the level of chitobiase activity may be selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate. In still another aspect of this embodiment, the step of determining the level of chitiobiase activity comprises determining the level of *p*-nitrophenol released from a substrate. In still another aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing the host cells to a desired set of environmental conditions. In a further aspect of this embodiment, the step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting the host cells with a compound to be tested for its influence on the level of transription from siad regulartory element. For example, the compound may comprise a compound to be tested for activity as a drug.

Brief Description of the Drawings

Figure 1 illustrates plasmids pJMF3 and pJMF4 containing *attP* in 2 different orientations and the *lac* promoter with the first 21 amino acids of *lacZα* (from pUC19) fused in-frame to the *chb* gene. The sequence of the fusion region is shown in Figure 2. Restriction sites shown are found once in the plasmid sequences except for *Not*I, which has 2 sites flanking the P15A origin.

Figure 2 illustrates the sequence of the *lac* promoter and the chitobiase fusion found in pJMF3 and pJMF4 (SEQ ID NOS: 15 and 16). Fusion between *lacZα* (from pUC19) and *chb* [Soto-Gill, R.W. et al, J. Biol. Chem. 264:14778-14782 (1998)] is indicated by (/); start of transcription is indicated by (+1). Sequence and binding sites in the *lac* promoter regulatory region are found in Dickson, R.C. et al., Science 187:27-32 (1975), the disclosure of which is incorporated herein by reference in its entirety. Restriction enzyme sites shown in Figure 2 are found once in the plasmid sequences except for *Sph*I, which has 2 sites; these different restriction sites can be used to replace

the *lac* promoter with another promoter together with part of a coding region to produce an in-frame fusion with *chb*.

Figure 3 depicts integration of *chb* fusions into the chromosome by site-specific recombination between *attB* and *attP*. The steps involved are described below and in Diederich, L.L, et al., Plasmid 28:14-24 (1992), the disclosure of which is incorporated herein by reference in its entirety.

Figure 4 illustrates plasmids pDYK9 and pDYK11 which contain *dnaA-chb* fusions. pDYK9 is deleted for the *rpmH* regulatory region. The orientation of *attP* in pDYK9 and pDYK11 is the same as that of pJMF3. After integration at *attB* of the larger *Not*I fragment, the *dnaA* promoters are oriented to transcribe in the same direction as replication fork movement.

Figure 5 illustrates the *rmpH-dnaA* regulatory region and *dnaA*-chitobiase fusion (SEQ ID NO: 17). The *dnaA* box and promoters are shaded, and the coding region of the *dnaA* gene is striped. The fusion contains two amino acids (between the backslashes) from pUC19. The region cloned into pDYK9 is between *Sph*I primer II and *Kpn*I primer, and the region cloned into pDYK11 is between *Sph*I primer I and *Kpn*I primer. The numbers above the primers refer to the nucleotide in the sequence amplified. For sequence numbering and the locations of promoters and protein binding sites, see Froelich, J.M. et al., J. Bacteriol. 178:6006-6012 (1996); Hansen, F.G. et al., EMBO J. 1:1043-1048 (1982) and Messer, W. and C.W. Weigel, Initiation of Chromosome Replication. p. 1579-1601 (1996) In F.C. Neidhart, R. Curtiss III, J.L. Ingraham, E.CC. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger (Eds.), Escherichia coli and Salmonella Cellular and Molecular Biology., ASM Press, Washington, D.C., the disclosures of which are incorporated herein by reference in their entireties.

Detailed Description of the Preferred Embodiment

Reporter genes and reporter gene constructs play a number of important roles in a variety of molecular biology techniques. For example, reporter genes may be used to determine whether a sequence contains a promoter or other cis-acting element which directs transcription, such as an enhancer. In addition, reporter genes may be used to identify regulatory sites in promoters or other cis-acting elements and to determine the

effects of mutating these regulatory sites on the level of gene expression directed by the promoters or other cis-acting elements. Reporter genes may also be used to detect successful transformation. In addition, reporter genes may be used to monitor gene expression under various conditions and to identify drugs.

The structure of a reporter gene construct containing the cytoplasmic form of chitobiase will vary according to its purposes. When the reporter gene construct is a vector, one must decide between a vector that incorporates the reporter gene into the host's genome or one that replicates extrachromosomally, such as a plasmid. When the reporter construct is not designed to integrate into the host genome, the vector can contain an origin of replication with activity in the host cell of interest. This feature provides the reporter gene vector the ability to replicate within the host cell in which it has been introduced. In addition to the origin of replication, reporter constructs often contain a promoter, a multiple cloning site, a selectable marker, and of course a reporter gene. Reporter constructs for use in eukaryotic cells may also contain a polyA site adjacent to the reporter gene.

Given the utility of reporter gene constructs, it is not surprising that a number of cytoplasmic reporter gene constructs and different reporter genes are available for use by those of skill in the art. For example, the cytoplasmic reporter enzymes chloramphenicol acetyltransferase (CAT), firefly luciferase, β-glucuronidase (GUS), green fluorescent protein (GFP), and ß-galactosidase have been used extensively. However, such reporters all have individual shortcomings that may limit or preclude their usage under some conditions. For example, high levels of GFP are toxic to the cell. In addition, reporter enzymes are not expressed equally in all cell types nor are they equally stable when expressed in all cell types. Furthermore, there is a recognized need for multiple reporter enzymes that can be assayed independently of one another in order to simultaneously study the regulation of multiple genes within a single cell type. Therefore, there exists a continuing need to identify reporter enzymes with useful properties.

The cytoplasmic enzyme ß-galactosidase is widely used as a reporter gene in various microbiological and molecular biological studies. This enzyme is used in both *in vitro* and *in vivo* assays. The wide acceptance of this reporter system results, in part, because it is non-isotopic and extremely flexible. It is used in a number of assay

formats and has an extremely broad linear range. Nevertheless, because ß-galactosidase is present in the cytoplasm of various host cells such as *Escherichia coli*, deletion of the *lacZ* gene, the source of the enzyme, is often required prior to its use in a host cell system. One goal of the present invention was to provide an alternative intracellular enzyme for use as a reporter.

An extensive discussion of various molecular biology techniques is available in Ausubel, et al., (eds) "Short Protocols in Molecular Biology," Wiley and Sons, Inc., New York (1997), the disclosures of which are incorporated herein by reference in their entireties. Examples of such techniques include isolating and preparing DNA for manipulation, gel electrophoresis, polymerase chain reaction (PCR), determining nucleic acid sequences, screening nucleic acid libraries, mutagenesis of DNA, and introducing DNA into host cells.

The structure of a reporter gene construct will vary according to its purposes. The reporter gene constructs are constructed according to standard techniques of molecular biology well known in the art.

When the reporter gene construct is a vector, one must decide between a vector that incorporates the reporter gene into the host's genome or one that replicates extrachromosomally, such as a plasmid. When integration of the reporter gene is a desired result, a reporter gene construct will contain sequences that will facilitate incorporation.

One example of integration sequences that can be included in a reporter gene construct is the λ *attP* site. This site permits a single copy of the reporter gene to be incorporated into a host bacterial genome. Integration-promoting sequences with utility in mammalian cells include the long terminal repeats found in retroviral genomes. These sequences promote viral genome integration in a host genome and have been used extensively by those of skill in the art to promote the integration of exogenous sequences in mammalian host cells.

When the reporter construct is not designed to integrate into the host genome, it is common that the vector contain an origin of replication with activity in the host cell of interest. This feature provides the reporter gene vector the ability to replicate within the host cell in which it has been introduced.

In addition to the origin of replication, reporter constructs will often contain additional features that promote the expression of the nucleic acid sequence or sequences contained in the construct. These additional sequences can include a polyA site, a multiple cloning site, a drug resistance marker, and of course a reporter gene.

The present invention relates to the use of chitobiase as a reporter gene. The chitobiase may be used as a reporter in bacteria, plants, mammalian cells and other host cell lines. One possible alternative to using ß-galactosidase as a reporter gene was to develop cytoplasmic N,N'-diacetylchitobiase (*N*-acetyl-ß-D-glucosaminidase, EC 3.2.1.30) for use as a reporter enzyme. One advantage of the enzyme *N*-acetyl-ß-D-glucosaminidase or "chitobiase" over ß-galactosidase is that genes encoding chitobiase are missing from many bacteria, including *E. coli*, some fungi, and some eukaryotic cells. Thus, it is not necessary to engineer many host cells to lack reporter activity as is the case with ß-galactosidase. The present invention also relates to various protein expression vectors that can be used to express the reporter gene. In addition, the present invention may be used in conjunction with other reporter enzymes in systems in which the regulation or activities of multiple genes is to be studied simultaneously.

Chitobiase is one of two enzymes that hydrolyze chitin, an abundant insoluble polysaccharide, to its monomeric unit, *N*-acetylglucosamine (GlcNac). Chitobiase is known to be present in a number of organisms. For example, the chitobiase enzyme is known to be present in various genera including *Arabidopsis, Bacillus, Bombyx, Bos, Caenorhabditis, Candida, Dictyostelium, Entamoeba, Felis, Homo, Korat, Lactobacillus, Leishmania, Mus, Pisum, Porphyromonas, Pseudoalteromonas, Rattus, Serratia, Streptomyces, Sus, Trichoderma,* and *Vibrio*. Specific examples of organisms known to contain chitobiase include *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

One source of the enzyme is the marine bacterium, *Vibrio harveyi*. *Escherichia coli* cells harboring a plasmid carrying the *chb* gene from *Vibrio harveyi* were reported to produce the enzyme, which was found to be associated with the outer membrane of the bacterial cells. These are described in Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli.*," J. Bacteriol. *169*:3785-3791 (1987) and Soto-Gil & Zyskind, *N,N'*-Diacetylchitobiase of *Vibrio harveyi* primary structure, processing, and evolutionary relationships. J. Biol Chem. *264*:14778-14782 (1989), both of which are hereby incorporated by reference.

The present invention contemplates expressed cytoplasmic forms of chitobiase in various forms. In one embodiment, the signal sequence is deleted from the amino terminal portion of the protein. Presumably the removal of this sequence results in the expression of a cytoplasmic form of the enzyme that is not secreted from the host cell or incorporated into the membrane of the host cell producing the enzyme.

The present invention also contemplates the generation of fusion proteins comprising a fusion polypeptide joined in frame to chitobiase. Preferably, the fusion polypeptide comprises a polypeptide other than chitobiase, such as a heterologous protein. The heterologous polypeptide may comprise a polypeptide having a biological activity (such as an enzymatic or other activity besides activity as an immunogen) or the heterologous polypeptide may not have a biological activity. The heterologous polypeptide does not include a signal sequence which directs its secretion. Preferably the heterologous polypeptide is not β-galactosidase or a portion thereof. Thus, the fusion reporter gene construct contains a sequence encoding the fusion polypeptide genetically fused in frame with a sequence encoding chitobiase. In one embodiment, this fusion may remove the amino-terminal signal peptide sequence of chitobiase and replace it with a heterologous protein.

In another embodiment, the fusion protein construct comprises a chitobiase gene sequence that has been truncated to remove at least the signal peptide sequence of the gene. Alternatively, mutations may be introduced into the signal peptide sequence so that it is no longer functional. Such mutations may be introduced using a variety of techniques familiar to those skilled in the art, including site directed mutagenesis, cassette mutagenesis, and chemical mutagenesis.

Once the reporter gene construct is made it is introduced into a host cell line for testing. Host cells of prokaryotic and eukaryotic origin can be used with the reporter gene constructs of the present invention. A variety of methods are available to introduce reporter gene constructs into host prokaryotic cells. For example, bacteria can be transformed using calcium chloride, electroporation, or viral vectors such as the filamentous phages. These and other prokaryotic transformation protocols are well known in the art.

Alternatively, the sequence encoding chitobiase may be introduced in eukaryotic cells, including yeast, mammalian, plant, and insect cells. For example, the sequence encoding chitobiase may be inserted into a yeast artificial chromosome, a yeast plasmid, a bovine papilloma virus vector or other extrachromosomal vector, a retroviral vector, a Ti-plasmid, or a baculovirus vector. A variety of such vectors are known to those skilled in the art. The vectors may be introduced into any of the yeast, mammalian, plant, and insect cells familiar to those skilled in the art.

The introduction of the reporter gene construct into mammalian cells can likewise utilize a number of transfection protocols well known to those of skill in the art. As discussed above, transfections can be transient or stable. Examples of suitable transfer protocols include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection, and viral transfection. These and other eukaryotic transformation protocols are well known in the art.

Following introduction of the reporter gene construct into the host cell of interest, the enzymatic activity of the reporter gene is measured. Preferably, the chitobiase assays are performed after permeabilizing or lysing the host cells. There are a variety of cell permeabilization and cell lysis procedures available to those of ordinary skill in the art, including methods such as sonication or lysozyme treatment. One embodiment of the present invention uses toluene treatment to permeabilize cells. The details of this method are discussed in D.Y. Kalabat et al., BioTechniques 25:1030-1035 (1998) and Miller , J.H. A Short Course in Bacterial Genetics, CSH Laboratory Press, Cold Spring Harbor, NY 1992, the disclosures of which are incorporated herein by reference in their entireties.

Cellular chitobiase activity can be measured quantitatively by following the hydrolysis of chitobiase substrates. Examples of substrates with utility in chitobiase

activity assays include *N,N'*-diacetylchitobiose (chitobiose), *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminide (PNAG)(Sigma Chemical, St. Louis, MO), and 5-bromo-4-chloro-3-indolyl-*N*-acetyl-ß-D-glucosaminide (X-Gluc)(Sigma Chemical, St. Louis, MO). Other substrates are also contemplated for use in the assays of the present invention.

Products produced by the hydrolysis of the chitobiase substrates are monitored using various means familiar to those skilled in the art. For example, various optical means are known to those skilled in the art. One such optical means may comprise detection of chemiluminescent or fluorescent products released from a substrate. Alternatively, the level of chitobiase activity may be determined by measuring the amount of light absorbed by a product produced from a substrate or measuring a decrease in the amount of a detectable substrate. In one embodiment, *p*-nitrophenol is released from the substrate and measured at 400 nm. Other monitoring methods well known in the art can be used to quantitate signals produced in the chitobiase assay. These may inlcude use of radioactive substrates or substrates having radiofrequency tags. In another embodiment, blue/white colony indicator plates are used to monitor enzyme activity.

Another embodiment of the present invention is a kit. One aspect of this embodiment includes a reporter gene construct comprising a vector containing a chitobiase reporter gene. The reporter gene construct also contains a multiple cloning site containing a variety of restriction endonuclease cutting sites that facilitate the introduction of exogenous DNA into the construct.

The kit embodiment of the present invention also includes those components necessary to assay for chitobiase activity produced by the reporter gene construct. For example, in one embodiment, the kit will include a supply of a suitable chitobiase substrate whose metabolism into product by the reporter enzyme can be assayed.

EXAMPLES

The following Examples are disclosed to assist in the understanding of the present invention. The Examples below should not be construed to limit the scope of the invention and such variations of the invention now known or later developed, which would be within the purview of one of ordinary skill in the art and are considered to fall within the scope of the invention hereinafter claimed.

Example 1

Construction of Vectors

Vectors were constructed using polymerase chain reaction (PCR) products that were cloned first at the *Sma*I or *Eco*RV restriction sites of plasmid pBluescript II (pKSII+)(Stratagene; San Diego, CA). The nucleotide sequence of all PCR products was determined using standard techniques well known in the art. Unmethylated plasmid DNA was isolated from an *E. coli dam*$^-$ strain when cutting with the *Bcl*I enzyme was required.

The pDYK9 (SEQ ID NO: 11) plasmid was constructed by ligating a *Sph*I-*Kpn*I PCR product containing the *dnaA* promoter region into plasmid pRSG196 that contains the *V. harveyi* chitobiase gene (see Jannatipour, M., et al.). Briefly, the *V. harveyi* chitobiase gene was prepared by cloning a *Sau*3A partial digest of *V. harveyi* DNA into the single *Bam*HI site with the *tet* gene of pMK2004 [See Soto-Gill & Zyskind, "Cloning of *Vibrio harveyi* chitinase and chitobiase genes in *Escherichia coli*," pp. 209-223, *In* J.P. Zikakis (ed.), Chitin, chitosan, and related enzymes," Academic Press, Inc., New York (1984)]. The clones containing the gene of interest were detected by the presence of the yellow *p*-nitrophenylate product after individual colonies of the clone bank were sprayed with 10 mM PNAG dissolved in 100 mM sodium phosphate, pH 7.0. Restriction maps of the positive clones were made according to standard techniques well known in the art.

The *chb* gene was subcloned in a 3.5-kb *Eco*RI fragment from pRSG14 [See Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli.*," J. Bacteriol. *169*:3785-3791 (1987)] into the *Eco*RI site of pUC19 [Yanisch-Perron, c.J. et al., Gene 33: 103-119 (1985)]. Plasmid pRSG196 was constructed by deleting a 0.5-kb *Sst*I fragment from one of these clones [See Jannatipour, M. et al., "Translocation of *Vibrio harveyi N,N'*-diacetylchitobiase to the outer membrane of *Escherichia coli.*," J. Bacteriol. *169*:3785-3791 (1987)].

The pRSG196 plasmid was also cut with *Sph*I and *Kpn*I to accommodate the insertion of the *dnaA* fragment. Plasmid pAC17 [described in Chiaramello & Zyskind, "Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate," J. Bacteriol. 172:2013-2019 (1992) hereby incorporated by reference] served as template with primers 5'-GCA CAT GCA TGC TGG TCA TTA

AAT TTT CC-3' (SEQ ID NO 1) and 5'-CGG GGT ACC AAC TCA TCC TGC AAT CG-3' (SEQ ID NO 2) producing a PCR product 374 bp long that contains 353 bp from the *dnaA* promoter region [bases 583 to 935, numbering according to Hansen, F. G., et al., "The nucleotide sequence of the *dnaA* gene promoter and of the adjacent *rpmH* gene, coding for the ribosomal protein L34, of *Escherichia coli*," EMBO J. 1:1043-1048 (1982)]. The forward primer (*Sph*I primer II) contained an *Sph*I site and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning. This created an in-frame fusion between the amino-terminal 17 amino acids of DnaA and the carboxy-terminal end of chitobiase deleted for the amino-terminal 22 amino acids including the signal peptide.

The next step in constructing the pDYK9 vector was taking the 3270 bp *Dra*I-*Hin*dIII (partial digest) fragment containing the *dnaA-chb* fusion and ligating it to the pACYC184 2555 bp *Hin*cII-*Hin*dIII fragment carrying chloramphenicol-resistance (Cm^r) and the P15A origin. This fragment was described in Chang & Cohen, "Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid," J. Bacteriol. 134:1141-1156 (1987).

A *Not*I site was introduced at the *Acc*I site after digestion with *Acc*I, treatment with Mung bean nuclease, and ligation to phosphorylated *Not*I linkers (New England Biolabs, Inc.; Beverly, MA). A *Not*I site was introduced at the *Ase*I site after digestion with *Ase*I, treatment with Mung bean nuclease, and ligation to phosphorylated *Not*I linkers (New England Biolabs, Inc.; Beverly, MA). An *Xba*I-*Sph*I PCR product containing the *rrnBt1t2* terminator was ligated into this plasmid cut with the same enzymes creating pDYK7. *E. coli* chromosomal DNA served as template with primers 5'-CTA GTC TAG ATG CCG AAC TCA GAA GTG A-3' (SEQ ID NO 3) and 5'-GCA CAT GCA TGC GGG GGA TGG CTT GTA GAT-3' (SEQ ID NO 4) to produce a PCR product 357 bp long that contains bases 6534 to 6869 from the *rrnB* operon [numbering according to Brosius, J., et al., "Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*." J. Mol. Biol. 148:107-127 (1981)] and includes the complex transcription termination region of this operon which is described in Orosz, A., et al., "Analysis of the complex transcription termination region of the *Escherichia coli rrnB* gene," Eur. J. Biochem. *201*:653-659 (1991). The forward primer contained an *Xba*I site and the reverse primer contained an *Sph*I site.

A *Bcl*I-*Sma*I PCR product containing the λ *attP* site was ligated into pDYK7 cut with *Tth111*I, treated with Mung bean nuclease, then digested with *Bcl*I. Plasmid pHN894 [described in Goodman, S. D., et al., "Deformation of DNA during site-specific recombination of bacteriophage lambda: Replacement of IHF protein by HU protein or sequence-directed bends," Proc. Natl. Acad. USA *89*:11910-11914 (1992)] served as template with the forward primer 5'-CAT GAT CAT GCG ACA GGT TTG ATG A-3' (SEQ ID NO 5) and the reverse primer 5'-GGG GGC GCC TAC CTT TCA CGA G-3' (SEQ ID NO 6) producing a PCR product 466 bp long that contains the λ *attP* site. The 466 bp PCR product containing the λ *attP* site was first cloned into the *Sma*I site of the pKSII+ plasmid to produce pDYK8. The forward primer contains a *Bcl*I site and the reverse primer contains G's at the 5' end in order to recreate a *Sma*I site when cloning the PCR fragment into a *Sma*I site. This PCR product includes bases -211 to +241 from the center of the *attP* core and the sequence required for optimum λ *attP* site integration as described in Nagaraja & Weisberg, "Specificity determinants in the attachment sites of bacteriophages HK022 and λ," J. Bacteriol. 172:6540-6550 (1990). The orientation of *attP* is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *p2* promoters is the same as replication fork movement mimicking the orientation at the wild type *dnaA* promoters.

Vector pDYK11 (SEQ ID NO: 12) was constructed by ligating an *Sph*I-*Kpn*I PCR product containing the *rpmH-dnaA* promoter region into pDYK9 also digested with *Sph*I and *Kpn*I. Plasmid pAC17, described in Chiaramello & Zyskind, (1992), served as template with the primers 5'-CAT GCA TGC ATG AAA CGA TGG ACA CC-3' (SEQ ID NO 7) and 5'-CGG GGT ACC AAC TCA TCC TGC AAT CG-3' (SEQ ID NO 8) to produce a PCR product 616 bp long that contains 598 bp from the *rpmH-dnaA* regulatory region [bases 338 to 935, numbering according to Hansen, F. G., et al., (1982)]. The forward primer (*Sph*I primer I) contained an *Sph*I site and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning. SEQ ID NO: 18 provides the complete coding sequence of the DNA encoding the dnaA/chitobiase fusion protein. The complete sequence of the dnaA/chitobiase fusion protein is provided in SEQ ID NO: 19.

The vector pJMF3 (SEQ ID NO: 13) was constructed by first ligating an *Ase*I linker (5'-CATTAATGCATG-3' (SEQ ID NO 9) self-hybridized) into the *Sph*I site of

pDYK11. The pUC19, [described in Yanisch-Perron C., et al., "Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors," Gene *33*:103-19 (1985)] *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to this plasmid after digestion with *Ase*I and *Kpn*I. The resulting in-frame fusion between the amino-terminal 21 amino acids of the pUC19 *lacZ*(α) peptide [Yanisch-Perron C., et al., 1985] and the carboxy-terminal end of chitobiase deleted for the amino-terminal 22 amino acids is identical to the protein fusion in pRSG196 [Jannatipour, M., et al., (1995)].

Vector pJMF4 (SEQ ID NO: 14) was constructed by ligating the *Bam*HI-*Eco*RV fragment of pDYK8 containing the λ *attP* site into pDYK7 which had been digested with *Tth111*I, treated with Mung bean nuclease, then digested with *Bcl*I, to create pTKP9. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK9. The pDYK11 *Bsp*MII-*Kpn*I fragment containing the *rpmH-dnaA* promoter region was then ligated into pTKP9 digested with the same enzymes to create pTKP11. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK11. An *Ase*I linker (5'-CATTAATGCATG-3' (SEQ ID NO 10) self-hybridized) was ligated into the *Sph*I site of pTKP11 to create pJMF2. The pUC19 [as described in Yanisch-Perron C., et al., (1985)] *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to pJMF2 cut with *Ase*I and *Kpn*I to create pJMF4.

Example 2

Site-specific Recombination

To move the chitobiase fusions in pDYK9 and pDYK11 to the *attB* site in the chromosome, *Not*I fragments from these plasmids were self-ligated and transformed or electroporated into strain WM2269 (DH5α containing pLDR8) [See Zyskind, J. W. and S. I. Bernstein (1992) "Recombinant DNA Laboratory Manual," Academic Press, San Diego, CA]. Plasmid pLDR8, described in Diederich, L. L. J., et al., "New cloning vectors for integration into the λ attachment site *attB* of *Escherichia coli* chromosome," Plasmid *28*:14-24 (1992), expresses integrase from the λ P_R promoter and contains the λ cI_{857} repressor gene, a kanamycin resistance gene, and a temperature-sensitive origin of replication. The transformed or electroporated cells were incubated at 42°C with shaking for 30 min then moved to 37°C for 1 h followed by selection on Luria broth

agar plates containing chloramphenicol (25 μg/ml) at 42°C. Transformants were screened for loss of kanamycin resistance and, therefore, loss of pLDR8.

Example 3

Bacteriophage P1 Transduction

Transduction with P1 bacteriophage by the method of Zyskind & Bernstein, Recombinant DNA Laboratory Manual. Academic Press, San Diego, CA (1992) was used to construct strains and to confirm the chromosomal location of the *dnaA-chb* fusions. Cotransduction of Cm^r (carried by the fusion) and *galK* (linked to *attB*) indicated that Cm^r and *galK* are linked on the chromosomes of strains DYK9W, DYK9F, DYK11W, and DYK11F.

Example 4

p-Nitrophenol Chitobiase Activity Assay

Chitobiase activity is located in the cytoplasm when its signal peptide is replaced by fusion with another peptide. Accordingly, chitobiase assays are performed on permeabilized or lysed cells. A variety of permabilization or cell lysis protocols are available to liberate the enzyme from within a cell population being tested. One such protocol involves toluene-treated cells washed once with M9 salts according to the method of Miller, J.H. A Short Course in Bacterial Genetics, CSH Laboratory Press, Cold Spring Harbor, NY 1992, which is hereby incorporated by reference. The toluenized cells are placed in a chitobiase buffer (10 mM Tris-HCl, pH 8.0, and 0.5 M NaCl) and 666 μM PNAG, the chitobiase enzyme substrate. NaCl is included because chitobiase has approximately 80% of full activity in the absence of salt, with maximal activity occurring between 0.25 and 0.6 M NaCl. Toluenized cells (0.772 ml) are preincubated at 28°C and the reaction started with the addition of 0.228 ml PNAG (1 mg/ml). After incubation at 28°C, the reaction is stopped by the addition of 1 ml of 1 M Tris base. The release of *p*-nitrophenol is measured at 400 nm and turbidity at 550 nm. *p*-Nitrophenol release is measured immediately at 400 nm with a molar absorptivity of 1.8×10^3 liters mol^{-1} cm^{-1}. Units are calculated after subtracting the light scattering factor ($1.5 \times OD_{550}$) from OD_{400} of the sample. The normalizing factor of 1.5 was determined previously by measuring the light scattering ratio of bacteria at OD_{400} and OD_{550}. One unit of chitobiase activity is the amount of enzyme that catalyzes the formation of 1 pmol of *p*-nitrophenol per min at 28°C. For comparison to Miller units

of ß-galactosidase [described in Miller, J. H., A Short Course in Bacterial Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1992)], the units are normalized to 1 ml of culture at OD_{450}=1.

Example 5

Determination of Chitobiase Activity in Cells Containing Vectors pJM3 and pJM4

(Figure 1) contain the *lacPO* promoter with the first 21 amino acids of *lacZα* (from pUC19) fused in-frame to the *chb* gene. These plasmids also contain the λ phage *attP* recombination site in different orientations, the gene encoding chloramphenicol acetyltransferase (*cat*), and a ribosomal terminator, *rrnBt1t2*, inserted upstream of the *lac-chb* fusion to prevent read-through from other promoters. The chitobiase activity associated with these plasmids (Table 1) is high in the absence of IPTG because of titration of *lac* repressor expressed from a single copy chromosomal gene. Induction by IPTG is approximately 10-fold (Table 1).

Table 1. Chitobiase Activity of *lacZ-chb* Fusion[a]

	Chitobiase[b] (U[c])	
Plasmid	**-IPTG**	**+IPTG (1 mM)**
pJMF3 in DH5α	668 ± 45	9320 ± 347
pJMF4 in DH5α	788 ± 44	7188 ± 477

[a] Overnight cultures were diluted 1:1000 into 50 mL prewarmed LB and grown to OD_{450} = 0.1. 1 mM IPTG was added to half of the culture, and growth continued to OD_{450} = 0.3.
[b] Triplicate samples were assayed. Mean chitobiase activities are given with standard deviations.
[c] One unit of chitobiase activity is 1 pmol of *p*-nitrophenol/min at 28°C. Units given for 1 mL of culture at OD_{450} = 1.

The *lac* promoter can be replaced with another promoter and a fusion protein created with chitobiase by cutting with *Sph*I or *Ase*I and either *Sal*I, *Kpn*I, or *Sst*I (Figure 2). Fusions created with these vectors can be moved to the chromosome by site specific recombination at the λ *attB* site to permit single copy analysis of the activity of the promoter. The protocol, as described more fully in Diederich, et al., "New cloning vectors for integration into the λ attachment site *attB* of *Escherichia coli* chromosome,"

Plasmid *28*:14-24 (1992), involves two components, (i) a circular DNA containing the λ attachment site, *attP*, the promoter-*chb* gene fusion, and the *cat* gene, and (ii) a helper plasmid, pLDR8, which contains the *int* gene under the control of the temperature-sensitive repressor, *cI857* and a temperature-sensitive origin of replication. The plasmid is digested with *Not*I to remove the P15A origin, and the fragment containing the chitobiase fusion is self-ligated prior to transformation into cells containing pLDR8. Integration occurs by site-specific recombination between *attP* and *attB* (17.4 min on the *E. coli* chromosome, Figure 3).

Example 6

Use of the Chitobiase Reporter Enzyme to Study dnaA Gene regulation

Two plasmids, pDYK9 and pDYK11, discussed in the Examples above, were constructed to assess the regulation of the *dnaA* gene using chitobiase as a reporter enzyme. These plasmids differ by the absence of the *rpmH* regulatory region in pDYK9 (Figure 4). These fusions were moved from the plasmid to the chromosomal *attB* site for single copy analysis as described above. After transformation of strain WM2269 with the ligated DNA, integration occurred by site specific recombination between the *attP* and the *attB* sites. The orientation of *attP* in pDYK9 and pDYK11 is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *dnaAp2* promoters is the same as movement of the replication fork. This orientation is the same as at the *dnaA* wild type location. The genetic location of the fusions was confirmed in the Cm^r transformants by demonstrating cotransduction of Cm^r and *galK*.

The fusions created in strain WM2269 were moved by P1 transduction to MG1655, creating strain DYK9W with pDYK9 and strain DYK11W with pDYK11. Deletion of the *rpmH* promoters had very little effect (1.4-fold) on chitobiase activity (Table 2, compare lines 1 and 3)

Table 2. Chitobiase and β-Gal Activities of *dnaA-chb* and *dnaA-lacZ* Fusions in WT and *fis* Mutant Backgrounds

Strains	Chitobiase[a] (U[b])	β-Gal[c] (Miller U[d])
DYK11W *fis*+	30.0 ± 2.1	
DYK11F *fis*::985	80.0 ± 1.2	
DYK9W *fis*+	44.0 ± 2.1	
DYK9F *fis*::985	96.0 ± 3.5	
RB220 *fis*+		59.8 ± 7.9
TP220 *fis*::767		115.2 ± 7.2

[a] Triplicate samples were assayed during exponential growth. Mean chitobiase activities are given with standard deviation.
[b] One unit of chitobiase activity is 1 pmol of *p*-nitrophenol per min at 28°C. Units given for 1 mL of culture at $OD_{450} = 1$.
[c] Data from Reference 8.
[d] Unit defined in Reference 13.

Fis protein binds to a site in the *dnaAp2* promoter that covers the -35 sequence (Figure 5), and appears to be a repressor of DnaA expression. A fusion protein with ß-galactosidase activity that is expressed from the *rpmH-dnaA* regulatory region has increased ß-galactosidase activity (1.9-fold) in a *fis*⁻ mutant when compared to Fis wild type cells [as described in Froelich, J. M., et al., "Fis binding in the *dnaA* operon promoter region," J. Bacteriol. *178*:6006-6012 (1996), data shown in Table 3]. Similarly, the absence of Fis leads to a greater than 2-fold increase in chitobiase activity of the DnaA-chitobiase fusion protein for the DYK9F and DYK11F strains, comparable in extent to that observed with the *dnaA-lacZ* fusion strain, TP220 (Table 3).

In the reporter gene constructs discussed in the Example, all upstream transcriptional activity was prevented from entering the *chb* reporter gene. The plasmid vectors, pJMF3 and pJMF4, described in the Examples above, contained the *rrnbt1t2* terminator upstream of the promoter fusion, which prevented readthrough from chromosomal promoters near the insertion site. Only chitobiase activity originating from the promoters of interest was expressed.

The *attP* site in these vectors allowed integration at the chromosomal *attB* in a specific orientation, depending on the vector used. With these vectors, any chitobiase fusion involving an essential gene can be moved to the chromosome, thus permitting single copy analysis with a chromosomal orientation similar to the wild-type gene.

Example 7

Identification of Promoters in Test Sequences

A nucleic acid prospectively containing a promoter is inserted upstream of a nucleic acid encoding a cytoplasmic form of chitobiase as described above. For example, the nucleic acid prospectively containing a promoter may be inserted into a restriction site in a sequence containing a plurality of restriction sites, such as a polylinker, which is located upstream of the nucleic acid encoding chitobiase. The test sequence may comprise any nucleic acid to be tested for promoter activity. In one embodiment, the test sequence may comprise a genomic DNA sequence. For example, the genomic DNA sequence may be a randomly generated DNA fragment, such as a fragment generated using shotgun cloning techniques, a restriction fragment, or any other sequence.

The vectors containing the test sequence upstream of the nucleic acid encoding chitobiase are introduced into an appropriate host cell. The level of chitobiase activity is assayed and compared to the level obtained from a control vector which lacks an insert in the cloning site. The presence of an elevated expression level in cell containing the vector containing the insert with respect to the level in cells containing the control vector without the insert indicates the presence of a promoter in the insert.

In some embodiments, the activity of the promoter in the test sequence may be assayed after exposure of the host cells to conditions which may influence the level of transcription from the promoter. For example, the environment of the host cells may be altered to determine whether the transcription level is influenced by environmental factors, including factors such as temperature, pH, nutrients, or availability of oxygen. In such analyses, chitobiase levels are assayed under a variety of environmental conditions to determine the effects of the environmental conditions on transcription levels from the promoter. In addition, the activity of the promoters may be examined in the presence or absence of compounds to be tested for regulatory activity. For example, the activity of the promoters may be tested by determining the levels of chitobiase produced in the presence or absence of compounds to be tested for activity as drugs.

Promoter sequences within the test sequences may be further defined by constructing nested deletions in the test sequences using conventional techniques such as Exonuclease III digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity as determined by measuring chitobiase activity in cells containing the deletion vectors. In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using techniques such as site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into the cloning sites in the promoter reporter vectors and measuring the levels of chitobiase produced from the mutated promoters.

The activity of known promoters may also be monitored by operably linking them to a nucleic acid encoding a cytoplasmic form of chitobiase. The activity of the promoters may be analyzed under various environmental conditions as described above. In addition, the activity of the promoters may be analyzed in the presence or absence of compounds to be tested for the ability to affect transcription from the promoters. For example, the compounds may be tested for activity as drugs.

In some embodiments, the chitobiase reporter constructs may be used in systems for identifying compounds that modulate cell surface protein-mediated activity or compounds which modulate the activities of intracellular signaling systems. Techniques for using reporter genes to identify compounds which modulate cell surface protein-mediated activity have been described in U.S. Patent Number 5,401,629 and U.S. Patent Number 5,436,128, the disclosures of which are incorporated herein by reference in their entireties. Briefly, in such methods, a construct comprising a promoter operably linked to a nucleic acid encoding a reporter enzyme is introduced into cells which express the cell surface protein and cells which do not express the cell surface protein. Each of the cells are contacted with test compounds and the effects of these compounds on transcription levels is measured by determining the level of activity of the reporter enzyme. The level of expression of the reporter gene in cells expressing the cell surface protein is compared to the level in cells which do not express the cell surface protein to identify compounds that modulate cell surface protein activity.

Similarly, the chitobiase reporter constructs may be used to identify compounds which influence the activity of intracellular signaling pathways, such as cAMP-based or phosphorylation-based pathways. In such methods, a promoter which is activated via such pathways is operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase. The cells are contacted with test compounds. Those compounds which activate the pathway to which the promoter responds will produce an enhanced level of chitobiase activity in the cells as compared to the level of chitobiase activity in control cells which have not been contacted with the test compound.

Example 8

Detecting Successful Transformation or Transfection Using Chitobiase

A vector comprising a sequence encoding a cytoplasmic form of chitobiase operably linked to a sequence capable of directing transcription of the chitobiase gene is introduced into a host cell. The host cells are contacted with a chitobiase substrate and those host cells which contain chitobiase activity are identified as cells which were successfully transformed or transfected. In some embodiments, a portion or replica of a colony may be lysed or permeabilized prior and the lysate or permeabilized cells may be contacted with the chitobiase substrate.

CONCLUSION

New gene reporter systems that use chitobiase have been described. Chitobiase has advantages over other reporter gene systems in that chitobiase is not found in many cell lines traditionally used in reporter gene systems.

Finally, the forgoing examples are not intended to limit the scope of the present invention, which is set forth in the following claims. In particular, various equivalents and substitutions will be recognized by those of ordinary skill in the art in view of the foregoing disclosure, and these are contemplated to be within the scope of the present invention. All references cited herein are incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for characterizing a promoter comprising:

providing a construct comprising said promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase;

introducing the construct into host cells; and

identifying sequences in said promoter which regulate transcription levels.

2. The method of Claim 1, wherein said cytoplasmic form of chitobiase lacks a signal sequence.

3. The method of Claim 2, wherein said nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, said fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide.

4. The method of Claim 1, wherein said nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus.*

5. The method of Claim 1, wherein said method of identifying sequences which are involved in directing transcription comprises mutagenizing said promoter.

6. The method of Claim 1, wherein said method of identifying sequences which are involved in transcription comprises constructing deletions in said promoter.

7. A method for identifying a regulatory element capable of directing or regulating transcription within a test nucleic acid sequence comprising:

providing a construct comprising said test nucleic acid sequence operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase;

introducing said construct into host cells; and

determining the level of chitobiase activity.

8. The method of Claim 7, wherein said cytoplasmic form of chitobiase lacks a signal sequence.

9. The method of Claim 8, wherein said nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, said fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide.

10. The method of Claim 7, wherein said nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei. Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

11. The method of Claim 7, wherein said reporter gene construct is introduced transiently.

12. The method of Claim 7, wherein said reporter gene construct is introduced stably.

13. The method of Claim 7, wherein said host cells are selected from the group consisting of prokaryotic cells and eukaryotic cells.

14. The method of Claim 7, further comprising permeabilizing or lysing said host cells.

15. The method of Claim 14, wherein said permeabilizing or lysing step comprises treating said host cells with toluene.

16. The method of Claim 7, wherein said step of determining the level of chitobiase activity is selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate.

17. The method of Claim 7, wherein said step of determining the level of chitiobiase activity comprises determining the level of *p*-nitrophenol released from a substrate.

18. The method of Claim 7, wherein said test nucleic acid sequence comprises a portion of genomic DNA.

19. The method of Claim 7, wherein said step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing said host cells to a desired set of environmental conditions.

20. The method of Claim 7, wherein said step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting said host cells with a compound to be tested for its influence on the level of transription from siad regulartory element.

21. A method of detecting successful transformation, comprising the steps of:

introducing a nucleic acid encoding a cytoplasmic form of chitobiase into host cells; and

detecting chitobiase expression in said host cells.

22. A fusion protein-reporter gene construct comprising a promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase fused in frame with a nucleic acid encoding a heterologous polypeptide, wherein said heterologous polypeptide is not β-galactosidase or a portion thereof and wherein said heterologous polypeptide does not contain a signal peptide.

23. The nucleic acid of Claim 22, wherein said nucleic acid encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

24. The nucleic acid of Claim 22, further comprising a λ site-specific recombination sequence.

25. A reporter gene construct comprising plasmid pJMF3.

26. A reporter gene construct comprising plasmid pJMF4.

27. A reporter gene construct comprising plasmid pDYK9.

28. A reporter gene construct comprising plasmid pDYK11.

29. A host cell comprising the construct of Claim 22.

30. The host cell of Claim 29 wherein said nucleic acid is integrated into a chromosome of said cell.

31. The host cell of Claim 29, wherein said nucleic acid is transiently expressed in said host cell.

32. A nucleic acid encoding a cytoplasmic form of chitobiase in which the signal sequence of native chitobiase has been inactivated or deleted.

33. The nucleic acid of Claim 32, wherein the signal sequence has been mutated to inactivate it.

34. An isolated or purified polypeptide comprising a cytoplasmic form of chitobiase fused in frame with a heterologous polypeptide, wherein said heterologous polypeptide is not β-galactosidase or a portion thereof and wherein said heterologous polypeptide does not contain a signal peptide.

35. An isolated or purified polypeptide comprising a cytoplasmic form of chitobiase in which the signal peptide of native chitobiase has been inactivated or deleted.

36. The polypeptide of Claim 35, wherein the signal sequence has been mutated to inactivate it.

37. A method for monitoring the activity of a promoter comprising:

providing a construct comprising said promoter operably linked to a nucleic acid encoding a cytoplasmic form of chitobiase;

introducing said construct into host cells; and

determining the level of chitobiase activity.

38. The method of Claim 37, wherein said cytoplasmic form of chitobiase lacks a signal sequence.

39. The method of Claim 38, wherein said nucleic acid encoding a cytoplasmic form of chitobiase encodes a fusion protein, said fusion protein comprising a cytoplasmic form of chitobiase fused to a heterologous polypeptide.

40. The method of Claim 37, wherein said nucleic acid encoding a cytoplasmic form encodes a cytoplasmic form of chitobiase obtained from an organism selected from the group consisting of *Alteromonas sp. 0-7, Arabidopsis thaliana, Bacillus subtilis, Bombyx mori, Bos taurus, Caenorhabditis elegans, Candida albicans, Dictyostelium discoideum, Entamoeba histolytica, Felis catus, Homo sapiens, Korat cats, Lactobacillus casei, Leishmania donovani, Mus musculus, Pisum sativum, Porphyromonas gingivalis, Pseudoalteromonas sp. S9, Rattus norvegicus, Serratia marcescens, Streptomyces plicatus, Streptomyces thermoviolaceus, Sus scrofa, Trichoderma harzianum, Vibrio furnissii, Vibrio harveyi , Vibrio parahaemolyticus,* and *Vibrio vulnificus*.

41. The method of Claim 37, wherein said reporter gene construct is introduced transiently.

42. The method of Claim 37, wherein said reporter gene construct is introduced stably.

43. The method of Claim 37, wherein said host cells are selected from the group consisting of prokaryotic cells and eukaryotic cells.

44. The method of Claim 37, further comprising permeabilizing or lysing said host cells.

45. The method of Claim 44, wherein said permeabilizing or lysing step comprises treating said host cells with toluene.

46. The method of Claim 37, wherein the step of determining the level of chitobiase activity is selected from the group consisting of measuring the amount of a chemiluminescent product produced from a substrate, measuring the amount of a fluorescent product produced from a substrate, measuring the amount of light absorbed by a product produced from a substrate and measuring a decrease in the amount of a detectable substrate.

47. The method of Claim 37, wherein said step of determining the level of chitiobiase activity comprises determining the level of *p*-nitrophenol released from a substrate.

48. The method of Claim 37, wherein said step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after exposing said host cells to a desired set of environmental conditions.

49. The method of Claim 37, wherein said step of determining the level of chitobiase activity comprises determining the level of chitobiase activity after contacting said host cells with a compound to be tested for its influence on the level of transription from siad regulartory element.

50. The method of Claim 49, wherein said compound comprises a compound to be tested for activity as a drug.

0360260

CHITOBIASE AS A REPORTER ENZYME

Abstract of the Disclosure

The present invention relates to reporter gene constructs encoding a cytoplasmic form of chitobiase (N,N'-diacetylchitobiase) and methods of using these reporter gene constructs. The use of a cytoplasmic form of chitobiase as a reporter enzyme is generally applicable to the study of gene expression in organisms which do not contain N-acetyl-ß-D-glucosaminidases.

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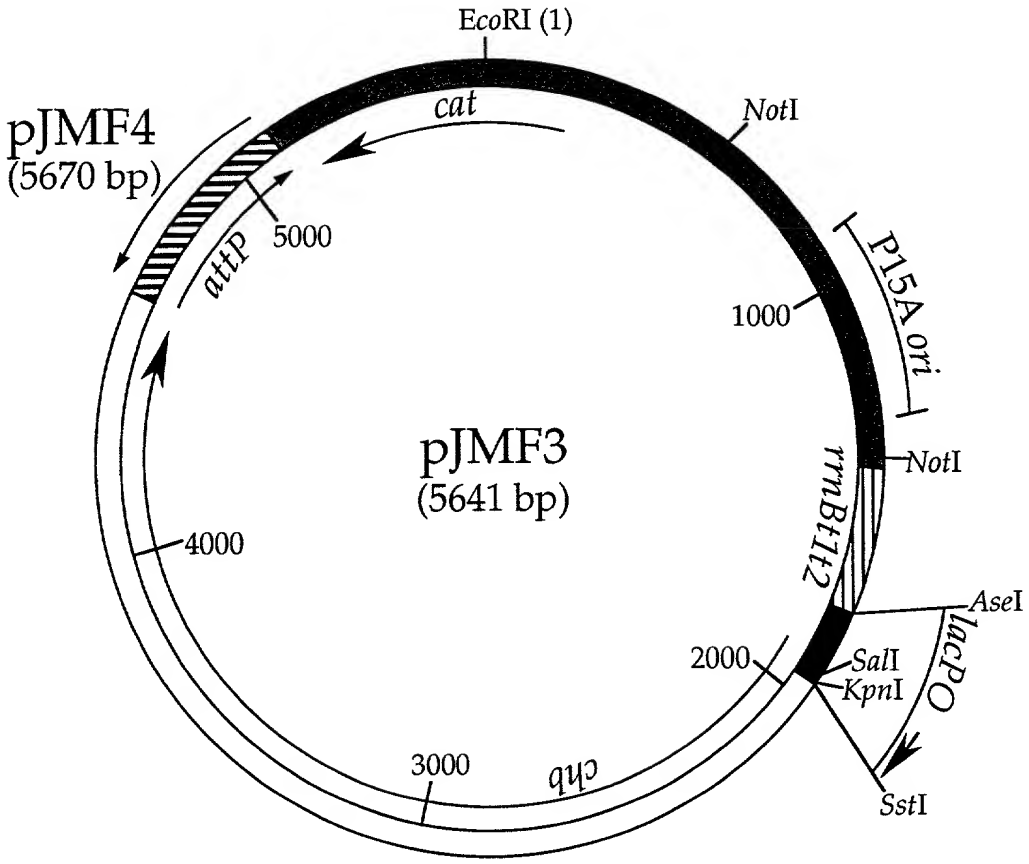
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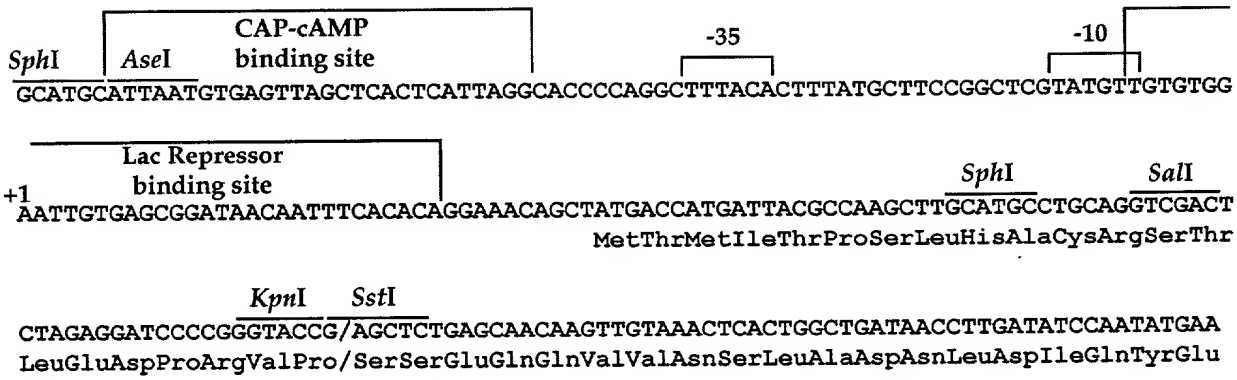

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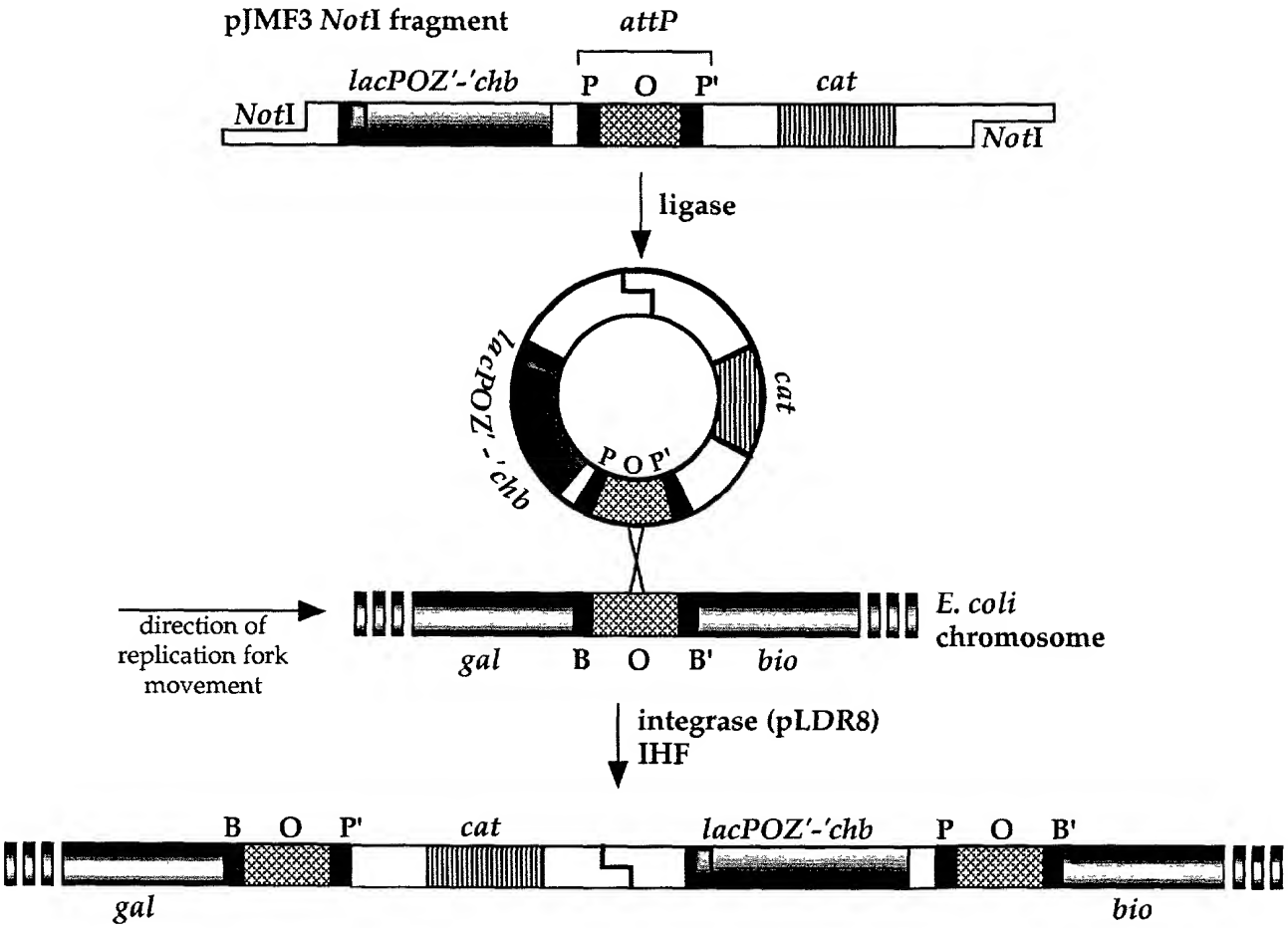
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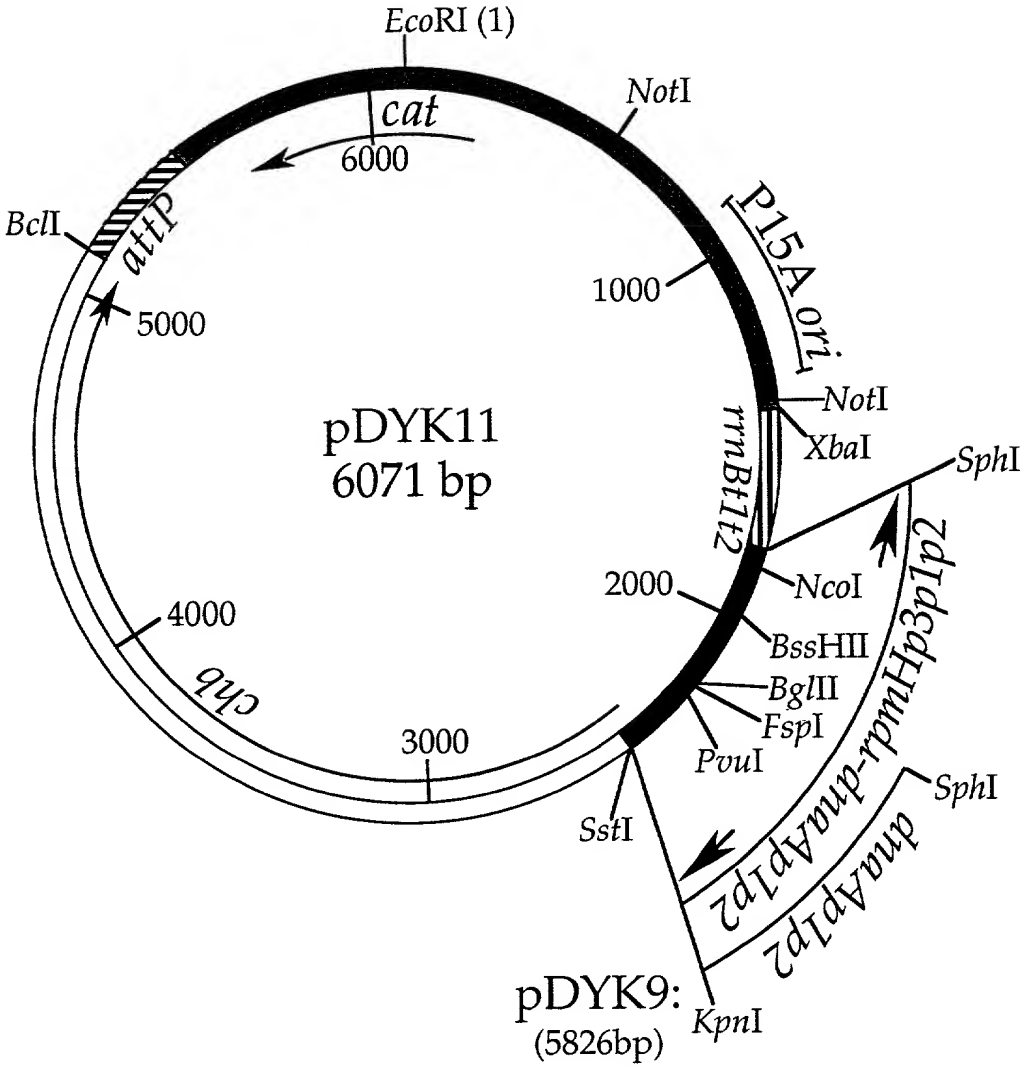
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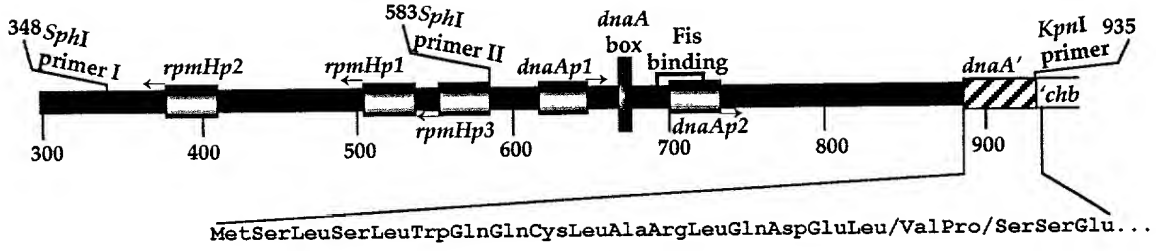

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09/09/2004 10:56:56

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<223> LacZ(/chitobiase Fusion

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atg acc atg att acg cca agc ttg cat gcc tgc agg tcg act cta gag 166
Met Thr Met Ile Thr Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu
 1 5 10 15

gat ccc cgg gta ccg agc tct gag caa caa gtt gta aac tca ctg gct 214
Asp Pro Arg Val Pro Ser Ser Glu Gln Gln Val Val Asn Ser Leu Ala
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Asp Asn Leu Asp Ile Gln Tyr Glu
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Leu Val Pro Ser Ser Glu
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<210> 18
<211> 2643

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<400> 18

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ttg gta ccg agc tct gag caa caa gtt gta aac tca ctg gct gat aac       96
Leu Val Pro Ser Ser Glu Gln Gln Val Val Asn Ser Leu Ala Asp Asn
             20                  25                  30

ctt gat atc caa tat gaa gtg tta act aac cat ggt gct aac gaa ggt      144
Leu Asp Ile Gln Tyr Glu Val Leu Thr Asn His Gly Ala Asn Glu Gly
         35                  40                  45

ctt gcg tgc caa gat atg ggc gca gaa tgg gct tct tgt aac aaa gta      192
Leu Ala Cys Gln Asp Met Gly Ala Glu Trp Ala Ser Cys Asn Lys Val
     50                  55                  60

aac atg acg ctt gtt aac caa ggt gaa gct gtt gac tca aaa gat tgg      240
Asn Met Thr Leu Val Asn Gln Gly Glu Ala Val Asp Ser Lys Asp Trp
 65                  70                  75                  80

gct att tac ttc cac agc att cgt ctg att ctg gat gtt gac aac gag      288
Ala Ile Tyr Phe His Ser Ile Arg Leu Ile Leu Asp Val Asp Asn Glu
                 85                  90                  95

cag ttc aaa atc tct cgt gta acg ggt gac cta cat aag cta gaa cca      336
Gln Phe Lys Ile Ser Arg Val Thr Gly Asp Leu His Lys Leu Glu Pro
            100                 105                 110

aca gat aag ttt gac ggc ttc gct gcc ggt gaa gag gtt gtt ctt cca      384
Thr Asp Lys Phe Asp Gly Phe Ala Ala Gly Glu Glu Val Val Leu Pro
        115                 120                 125

ttg gtt ggt gaa tac tgg caa cta ttt gaa act gac ttc atg ccg ggt      432
Leu Val Gly Glu Tyr Trp Gln Leu Phe Glu Thr Asp Phe Met Pro Gly
    130                 135                 140

gca ttc gtt tct gct cca aac gca gaa cct aag atg att gct tct cta      480
Ala Phe Val Ser Ala Pro Asn Ala Glu Pro Lys Met Ile Ala Ser Leu
145                 150                 155                 160

aat act gaa gat gtt gcg tct ttt gtg acg ggt ctt gaa ggt aac aac      528
Asn Thr Glu Asp Val Ala Ser Phe Val Thr Gly Leu Glu Gly Asn Asn
                165                 170                 175

cta aaa cgt aca cca gat gac aac aat gta ttt gca aac gct gtg tct      576
Leu Lys Arg Thr Pro Asp Asp Asn Asn Val Phe Ala Asn Ala Val Ser
            180                 185                 190
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cgt ttt gag aaa aac gaa gac cta gca aca caa gac gta tca acc acg      624
Arg Phe Glu Lys Asn Glu Asp Leu Ala Thr Gln Asp Val Ser Thr Thr
        195                 200                 205

tta cta cca aca cca atg cac gtt gaa gcg ggt aaa ggc aaa gta gat      672
Leu Leu Pro Thr Pro Met His Val Glu Ala Gly Lys Gly Lys Val Asp
    210                 215                 220

atc gcg gat ggt att gcg ctg cct aaa gac gca ttc gat gcg act cag      720
Ile Ala Asp Gly Ile Ala Leu Pro Lys Asp Ala Phe Asp Ala Thr Gln
225                 230                 235                 240

ttc gca gcg att caa gat cgt gca gaa gtg gta ggt gtg gac gtt cgt      768
Phe Ala Ala Ile Gln Asp Arg Ala Glu Val Val Gly Val Asp Val Arg
                245                 250                 255

ggt gat ctt cct gta agc atc act gtt gtt cct gca gac ttc acc ggt      816
Gly Asp Leu Pro Val Ser Ile Thr Val Val Pro Ala Asp Phe Thr Gly
            260                 265                 270

gaa tta gca aaa tct ggt gct tac gaa atg agc atc aaa ggc gac ggt      864
Glu Leu Ala Lys Ser Gly Ala Tyr Glu Met Ser Ile Lys Gly Asp Gly
        275                 280                 285

att gtg att aaa gcg ttc gac caa gca ggc gct ttc tac gca gta caa      912
Ile Val Ile Lys Ala Phe Asp Gln Ala Gly Ala Phe Tyr Ala Val Gln
    290                 295                 300

tct atc ttt ggc ctg gta gat agc caa aat gct gat tct cta cca caa      960
Ser Ile Phe Gly Leu Val Asp Ser Gln Asn Ala Asp Ser Leu Pro Gln
305                 310                 315                 320

ctg tct att aaa gat gcg cct cgt ttt gat tac cgt ggt gtg atg gtg     1008
Leu Ser Ile Lys Asp Ala Pro Arg Phe Asp Tyr Arg Gly Val Met Val
                325                 330                 335

gat gtg gct cgt aac ttc cac tct aag gac gca atc ctt gca acg cta     1056
Asp Val Ala Arg Asn Phe His Ser Lys Asp Ala Ile Leu Ala Thr Leu
            340                 345                 350

gac caa atg gca gcg tac aag atg aac aaa ctt cac ctt cac cta acc     1104
Asp Gln Met Ala Ala Tyr Lys Met Asn Lys Leu His Leu His Leu Thr
        355                 360                 365

gat gat gaa ggc tgg cgt tta gaa atc ccg ggt ctg cct gag ctg aca     1152
Asp Asp Glu Gly Trp Arg Leu Glu Ile Pro Gly Leu Pro Glu Leu Thr
    370                 375                 380

gaa gtg ggt gct aac cgt tgt ttc gat aca caa gag aaa agc tgt tta     1200
Glu Val Gly Ala Asn Arg Cys Phe Asp Thr Gln Glu Lys Ser Cys Leu
385                 390                 395                 400

ctg cct cag ctt ggc tct ggt cca acg aca gac aac ttt ggc tct ggc     1248
Leu Pro Gln Leu Gly Ser Gly Pro Thr Thr Asp Asn Phe Gly Ser Gly
                405                 410                 415
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tac ttc agc aaa gca gac tac gtg gaa atc ttg aaa tac gcg aaa gca     1296
Tyr Phe Ser Lys Ala Asp Tyr Val Glu Ile Leu Lys Tyr Ala Lys Ala
            420                 425                 430

cgt aac att gaa gtg att cca gaa atc gat atg cca gct cac gct cgt     1344
Arg Asn Ile Glu Val Ile Pro Glu Ile Asp Met Pro Ala His Ala Arg
        435                 440                 445

gca gca gta gta tca atg gaa gct cgt tac gac cgc cta atg gaa gaa     1392
Ala Ala Val Val Ser Met Glu Ala Arg Tyr Asp Arg Leu Met Glu Glu
    450                 455                 460

ggt aaa gaa gct gaa gcg aac gaa tac cgt ctg atg gat cct caa gat     1440
Gly Lys Glu Ala Glu Ala Asn Glu Tyr Arg Leu Met Asp Pro Gln Asp
465                 470                 475                 480

aca tca aac gta acg acg gtt cag ttc tac aat aag caa agc ttc atc     1488
Thr Ser Asn Val Thr Thr Val Gln Phe Tyr Asn Lys Gln Ser Phe Ile
                485                 490                 495

aac cca tgt atg gaa tct tca act cgc ttt gtt gat aag gtg att tca     1536
Asn Pro Cys Met Glu Ser Ser Thr Arg Phe Val Asp Lys Val Ile Ser
            500                 505                 510

gaa gtg gca gca atg cac caa gaa gct ggc gct cca cta aca act tgg     1584
Glu Val Ala Ala Met His Gln Glu Ala Gly Ala Pro Leu Thr Thr Trp
        515                 520                 525

cac ttc ggt ggt gac gaa gcg aag aac atc aag cta ggt gct ggt ttc     1632
His Phe Gly Gly Asp Glu Ala Lys Asn Ile Lys Leu Gly Ala Gly Phe
    530                 535                 540

caa gac gtt aac gca gaa gat aaa gta agc tgg aaa ggc acg att gac     1680
Gln Asp Val Asn Ala Glu Asp Lys Val Ser Trp Lys Gly Thr Ile Asp
545                 550                 555                 560

ctg tct aaa caa gac aag ccg ttt gca cag tct cca caa tgt cag acg     1728
Leu Ser Lys Gln Asp Lys Pro Phe Ala Gln Ser Pro Gln Cys Gln Thr
                565                 570                 575

cta atc aca gat ggc aca gtc agt gac ttt gct cac cta cca agc cac     1776
Leu Ile Thr Asp Gly Thr Val Ser Asp Phe Ala His Leu Pro Ser His
            580                 585                 590

ttc gcg gaa gaa gtg tcg aag att gtt gct gag aaa ggc att cca aac     1824
Phe Ala Glu Glu Val Ser Lys Ile Val Ala Glu Lys Gly Ile Pro Asn
        595                 600                 605

ttc caa gct tgg caa gat ggt ttg aaa tac agt gac ggc gaa aaa gcg     1872
Phe Gln Ala Trp Gln Asp Gly Leu Lys Tyr Ser Asp Gly Glu Lys Ala
    610                 615                 620

ttc gct aca gaa aat act cgc gta aac ttc tgg gac gtt ctg tac tgg     1920
Phe Ala Thr Glu Asn Thr Arg Val Asn Phe Trp Asp Val Leu Tyr Trp
625                 630                 635                 640

ggc ggt act tcc tca gtg tac gag tgg tct aag aaa ggt tac gac gtg     1968
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Gly Gly Thr Ser Ser Val Tyr Glu Trp Ser Lys Lys Gly Tyr Asp Val
                645                 650                 655

att gtt tct aac cca gat tac gtg tac atg gat atg cca tac gaa gtt     2016
Ile Val Ser Asn Pro Asp Tyr Val Tyr Met Asp Met Pro Tyr Glu Val
            660                 665                 670

gac ccg aaa gag cgt ggt tac tac tgg gca aca cgt gca acg gat act     2064
Asp Pro Lys Glu Arg Gly Tyr Tyr Trp Ala Thr Arg Ala Thr Asp Thr
        675                 680                 685

cgt aag atg ttt ggc ttt gca cca gag aac atg cct caa aac gca gaa     2112
Arg Lys Met Phe Gly Phe Ala Pro Glu Asn Met Pro Gln Asn Ala Glu
    690                 695                 700

act tct gta gat cgc gat ggc aat ggc ttt act ggt aaa ggt gaa atc     2160
Thr Ser Val Asp Arg Asp Gly Asn Gly Phe Thr Gly Lys Gly Glu Ile
705                 710                 715                 720

gaa gcg aaa cct ttc tac ggt cta tct gca caa ctt tgg tct gag aca     2208
Glu Ala Lys Pro Phe Tyr Gly Leu Ser Ala Gln Leu Trp Ser Glu Thr
                725                 730                 735

gta cgt aac gac gag caa tac gag tac atg gta ttc cct cgc gtc ctc     2256
Val Arg Asn Asp Glu Gln Tyr Glu Tyr Met Val Phe Pro Arg Val Leu
            740                 745                 750

gct gct gct cag cgt gca tgg cac cgt gct gac tgg gaa aac gac tac     2304
Ala Ala Ala Gln Arg Ala Trp His Arg Ala Asp Trp Glu Asn Asp Tyr
        755                 760                 765

aaa gtt ggt gtt gag tac tcg caa aac tct aat cta gtt gat aaa gca     2352
Lys Val Gly Val Glu Tyr Ser Gln Asn Ser Asn Leu Val Asp Lys Ala
    770                 775                 780

tcg cta aac caa gac tac aac cgc ttt gcg aac gta ctt ggt caa cgt     2400
Ser Leu Asn Gln Asp Tyr Asn Arg Phe Ala Asn Val Leu Gly Gln Arg
785                 790                 795                 800

gaa ctg gct aag cta gaa aaa tca ggt att gac tac cgc cta cca gta     2448
Glu Leu Ala Lys Leu Glu Lys Ser Gly Ile Asp Tyr Arg Leu Pro Val
                805                 810                 815

cca ggt gca aaa gta gaa gat ggt aag cta gca atg aac gtt cag ttc     2496
Pro Gly Ala Lys Val Glu Asp Gly Lys Leu Ala Met Asn Val Gln Phe
            820                 825                 830

cct ggc gta acg ctt caa tac tct ctg gat ggt gag aac tgg ttg act     2544
Pro Gly Val Thr Leu Gln Tyr Ser Leu Asp Gly Glu Asn Trp Leu Thr
        835                 840                 845

tat gca gac aac gct cgt cca aat gta act ggt gaa gtc ttc atc cgc     2592
Tyr Ala Asp Asn Ala Arg Pro Asn Val Thr Gly Glu Val Phe Ile Arg
    850                 855                 860

tcg gta tct gcg aca ggt gag aag gta agc cgt atc act agc gtg aaa     2640
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Substitute Sheet (Rule 26)

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taa                                                             2643
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Leu Asp Ile Gln Tyr Glu Val Leu Thr Asn His Gly Ala Asn Glu Gly
        35                  40                  45
Leu Ala Cys Gln Asp Met Gly Ala Glu Trp Ala Ser Cys Asn Lys Val
    50                  55                  60
Asn Met Thr Leu Val Asn Gln Gly Glu Ala Val Asp Ser Lys Asp Trp
65                  70                  75                  80
Ala Ile Tyr Phe His Ser Ile Arg Leu Ile Leu Asp Val Asp Asn Glu
                85                  90                  95
Gln Phe Lys Ile Ser Arg Val Thr Gly Asp Leu His Lys Leu Glu Pro
            100                 105                 110
Thr Asp Lys Phe Asp Gly Phe Ala Ala Gly Glu Glu Val Val Leu Pro
        115                 120                 125
Leu Val Gly Glu Tyr Trp Gln Leu Phe Glu Thr Asp Phe Met Pro Gly
    130                 135                 140
Ala Phe Val Ser Ala Pro Asn Ala Glu Pro Lys Met Ile Ala Ser Leu
145                 150                 155                 160
Asn Thr Glu Asp Val Ala Ser Phe Val Thr Gly Leu Glu Gly Asn Asn
                165                 170                 175
Leu Lys Arg Thr Pro Asp Asp Asn Asn Val Phe Ala Asn Ala Val Ser
            180                 185                 190
Arg Phe Glu Lys Asn Glu Asp Leu Ala Thr Gln Asp Val Ser Thr Thr
        195                 200                 205
Leu Leu Pro Thr Pro Met His Val Glu Ala Gly Lys Gly Lys Val Asp
    210                 215                 220
Ile Ala Asp Gly Ile Ala Leu Pro Lys Asp Ala Phe Asp Ala Thr Gln
225                 230                 235                 240
Phe Ala Ala Ile Gln Asp Arg Ala Glu Val Val Gly Val Asp Val Arg
                245                 250                 255
Gly Asp Leu Pro Val Ser Ile Thr Val Val Pro Ala Asp Phe Thr Gly
            260                 265                 270
Glu Leu Ala Lys Ser Gly Ala Tyr Glu Met Ser Ile Lys Gly Asp Gly
        275                 280                 285
Ile Val Ile Lys Ala Phe Asp Gln Ala Gly Ala Phe Tyr Ala Val Gln
    290                 295                 300
Ser Ile Phe Gly Leu Val Asp Ser Gln Asn Ala Asp Ser Leu Pro Gln
305                 310                 315                 320
Leu Ser Ile Lys Asp Ala Pro Arg Phe Asp Tyr Arg Gly Val Met Val
                325                 330                 335
Asp Val Ala Arg Asn Phe His Ser Lys Asp Ala Ile Leu Ala Thr Leu
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            340                 345                 350
Asp Gln Met Ala Ala Tyr Lys Met Asn Lys Leu His Leu His Leu Thr
        355                 360                 365
Asp Asp Glu Gly Trp Arg Leu Glu Ile Pro Gly Leu Pro Glu Leu Thr
    370                 375                 380
Glu Val Gly Ala Asn Arg Cys Phe Asp Thr Gln Glu Lys Ser Cys Leu
385                 390                 395                 400
Leu Pro Gln Leu Gly Ser Gly Pro Thr Thr Asp Asn Phe Gly Ser Gly
                405                 410                 415
Tyr Phe Ser Lys Ala Asp Tyr Val Glu Ile Leu Lys Tyr Ala Lys Ala
            420                 425                 430
Arg Asn Ile Glu Val Ile Pro Glu Ile Asp Met Pro Ala His Ala Arg
        435                 440                 445
Ala Ala Val Val Ser Met Glu Ala Arg Tyr Asp Arg Leu Met Glu Glu
    450                 455                 460
Gly Lys Glu Ala Glu Ala Asn Glu Tyr Arg Leu Met Asp Pro Gln Asp
465                 470                 475                 480
Thr Ser Asn Val Thr Thr Val Gln Phe Tyr Asn Lys Gln Ser Phe Ile
                485                 490                 495
Asn Pro Cys Met Glu Ser Ser Thr Arg Phe Val Asp Lys Val Ile Ser
            500                 505                 510
Glu Val Ala Ala Met His Gln Glu Ala Gly Ala Pro Leu Thr Thr Trp
        515                 520                 525
His Phe Gly Gly Asp Glu Ala Lys Asn Ile Lys Leu Gly Ala Gly Phe
    530                 535                 540
Gln Asp Val Asn Ala Glu Asp Lys Val Ser Trp Lys Gly Thr Ile Asp
545                 550                 555                 560
Leu Ser Lys Gln Asp Lys Pro Phe Ala Gln Ser Pro Gln Cys Gln Thr
                565                 570                 575
Leu Ile Thr Asp Gly Thr Val Ser Asp Phe Ala His Leu Pro Ser His
            580                 585                 590
Phe Ala Glu Glu Val Ser Lys Ile Val Ala Glu Lys Gly Ile Pro Asn
        595                 600                 605
Phe Gln Ala Trp Gln Asp Gly Leu Lys Tyr Ser Asp Gly Glu Lys Ala
    610                 615                 620
Phe Ala Thr Glu Asn Thr Arg Val Asn Phe Trp Asp Val Leu Tyr Trp
625                 630                 635                 640
Gly Gly Thr Ser Ser Val Tyr Glu Trp Ser Lys Lys Gly Tyr Asp Val
                645                 650                 655
Ile Val Ser Asn Pro Asp Tyr Val Tyr Met Asp Met Pro Tyr Glu Val
            660                 665                 670
Asp Pro Lys Glu Arg Gly Tyr Tyr Trp Ala Thr Arg Ala Thr Asp Thr
        675                 680                 685
Arg Lys Met Phe Gly Phe Ala Pro Glu Asn Met Pro Gln Asn Ala Glu
    690                 695                 700
Thr Ser Val Asp Arg Asp Gly Asn Gly Phe Thr Gly Lys Gly Glu Ile
705                 710                 715                 720
Glu Ala Lys Pro Phe Tyr Gly Leu Ser Ala Gln Leu Trp Ser Glu Thr
                725                 730                 735
Val Arg Asn Asp Glu Gln Tyr Glu Tyr Met Val Phe Pro Arg Val Leu
            740                 745                 750
Ala Ala Ala Gln Arg Ala Trp His Arg Ala Asp Trp Glu Asn Asp Tyr
        755                 760                 765
Lys Val Gly Val Glu Tyr Ser Gln Asn Ser Asn Leu Val Asp Lys Ala
    770                 775                 780
Ser Leu Asn Gln Asp Tyr Asn Arg Phe Ala Asn Val Leu Gly Gln Arg
785                 790                 795                 800
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Glu Leu Ala Lys Leu Glu Lys Ser Gly Ile Asp Tyr Arg Leu Pro Val
                805                 810                 815
Pro Gly Ala Lys Val Glu Asp Gly Lys Leu Ala Met Asn Val Gln Phe
            820                 825                 830
Pro Gly Val Thr Leu Gln Tyr Ser Leu Asp Gly Glu Asn Trp Leu Thr
        835                 840                 845
Tyr Ala Asp Asn Ala Arg Pro Asn Val Thr Gly Glu Val Phe Ile Arg
    850                 855                 860
Ser Val Ser Ala Thr Gly Glu Lys Val Ser Arg Ile Thr Ser Val Lys
865                 870                 875                 880
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